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Innate immunity, oxidative stress and body indices of Eurasian perch *Perca fluviatilis* after two weeks of exposure to artificial light at night

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Abstract

Artificial light at night (ALAN) can disrupt biological rhythms of fish and other vertebrates by changing the light information of the nocturnal environment. Disrupted biorhythms can impair the immune system of vertebrates as it has been shown for conditions with continuous illumination or long-day photoperiod in many vertebrates, including fish. Nonetheless, this has not been shown so far for typical ALAN scenarios with high light intensities during day and low light intensities at night. Therefore, in this study, proxies for the innate immune system and oxidative stress as well as body indices of Eurasian perch *Perca fluviatilis* were measured under a wide range of intensities of nocturnal illumination. The authors found no changes in parameters of the innate immune system and no significant changes in proxies for oxidative stress after 2-week exposures to nocturnal illuminance ranging from 0.01 lx to 1 lx in one experiment or from 1 lx to 100 lx in a second experiment. A decrease in the hepato-somatic index at the highest tested light intensity of 100 lx compared to the dark control was the only significant difference in all parameters among treatments. After 2 weeks of exposure, ALAN does not seem to seriously challenge the innate immune system and seems to cause less oxidative stress than expected. The results of this study contradict the findings from other studies applying continuous illumination or long-day photoperiod and highlight the importance of further research in this field. Because ALAN represents a sustained modulation of the environment that may have cumulative effects over time, long-term studies are required for a better understanding of how ALAN modulates the health of fish.

KEYWORDS
ALAN, fish, freshwater, light pollution, non-specific immune system, skyglow

1 | INTRODUCTION

The health of fish is protected by physiological defence mechanisms against environmental or biological hazards, which can be critical for survival and reproductive success. The numerous components of the immune system and the antioxidative defence system of fish can display daily and seasonal rhythms as they are shaped by environmental factors, such as photoperiod and temperature (Bowden, 2008; Fortes-Silva *et al*., 2019; Hidalgo *et al*., 2017). Artificial light at night (ALAN) is an unprecedentedly increasing environmental change, which is

introduced by humans to the nocturnal environment (Hölker *et al*., 2010) and changes the information on nocturnal illumination and photoperiod, which may result in suboptimal functioning of the immune system of fish and other vertebrates (Bowden, 2008; Navara & Nelson, 2007). Artificial light that is scattered in the atmosphere creates a dim glow of the night sky over large areas even many kilometres away from the original light source (Jechow *et al*., 2020; Kyba *et al*., 2011). This phenomenon, referred to as skyglow, illuminates the nocturnal environment at low intensities, and because of scattering, it affects not only cities but also suburban areas including the surrounding waters (Hänel *et al*., 2018). Thereby, skyglow can extend periods of twilight and can even blur rhythms of lunar illumination, especially in cloudy nights (Jechow *et al*., 2020; Puschnig *et al*., 2014).

An impairment of the immune system of fish by ALAN has mainly been studied in terms of 24 h illumination (LL) with nocturnal light intensities equal to experimental daylight intensities, which has been reviewed for fish (Bowden, 2008) and for mammals and birds (Navara & Nelson, 2007). Effects of LL on the immune system of fish include increased lysozyme activity (Burgos *et al*., 2004), reduced peripheral leucocyte numbers (Valenzuela *et al*., 2007) or increased antibody levels (Melingen & Wergeland, 2002) in salmonids. Furthermore, a prolonged exposure to light by a photoperiod with 14 h light and 10 h darkness (14 L:10 D) decreased the respiratory burst of blood leucocytes in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) after 1 week exposure compared to a photoperiod with 12 L:12 D (Burgos *et al*., 2004). In this context, effects of melatonin on the immune system are typically discussed as well because the nocturnally produced hormone transduces the light/dark information to immune cells. Fish leucocytes possess melatonin receptors, indicating a mediating effect of melatonin on leucocyte numbers and functioning as it has been shown in carp *Cyprinus carpio* L. 1758 (Kepka *et al*., 2015).

On top of the modulation of leucocyte activity, melatonin also acts as an antioxidant and modulator for antioxidative enzymes (Carrillo-Vico *et al*., 2013; Reiter *et al*., 2000). The antioxidative potential of melatonin in fish has been reviewed recently by Esteban *et al*. (2013). ALAN potently reduced nocturnal melatonin secretion in Eurasian perch *Perca fluviatilis* L. 1758 at nocturnal intensities between 0.01 lx and 100 lx (Brüning *et al*., 2015; Kupprat *et al*., 2020) and in many teleost species melatonin was likewise reduced even at low intensities of nocturnal illumination below 1 lx (*e.g.* Brüning *et al*., 2018; Nikaido *et al*., 2009). Indeed, in most vertebrate taxa this dose-dependent suppression of melatonin by ALAN can be observed (Grubisic *et al*., 2019). Because of the lack of the antioxidative potential of melatonin, an increase in oxidative stress is likely at LL or exposure to ALAN (Navara & Nelson, 2007). Increased activities of superoxide dismutase (SOD) and catalase (CAT) among other antioxidative enzymes were measured in fish exposed to LL as compared to a 12 L:12 D photoperiod in several studies (Corona-Herrera *et al*., 2018; Sreejith *et al*., 2007) indicating an increase in oxidative stress. Some of these effects could be reversed by the administration of melatonin *in vivo* or *in vitro* (Shin *et al*., 2011; Sreejith *et al*., 2007). Furthermore, melatonin had immuno-enhancing effects on leucocytes of sea bass *Dicentrarchus labrax* (L. 1758) and sea bream *Sparus aurata* L. 1758 (Cuesta *et al*., 2008) as well as *C. carpio* (Kepka *et al*., 2015). These studies support the idea that melatonin is a main modulator of the innate immune system of fish.

The above-mentioned studies revealed effects on the immune system under LL or long-day photoperiod, with daylight intensities of hundreds to thousands of lux. This study is the first to investigate the effects of typical ALAN scenarios (bright daylight and dimly lit nights) on innate immunity and indicators for oxidative stress in fish. The authors of this study took an explorative approach by measuring several parameters associated with the health of *P. fluviatilis* in response to ALAN. Generally, the innate or non-specific immune system is the first line of defence against pathogens. Thus, the authors measured the lysozyme activity in the blood plasma and the respiratory burst activity of head kidney leucocytes. Furthermore, they assessed thiobarbituric acid reactive substances (TBARS) as a measure for lipid peroxidation, and activities of SOD as well as CAT from liver tissue as indirect proxies for oxidative stress. In addition, the condition factor (*K*), the splenosomatic index (I_S) and the hepato-somatic index (I_H) served as measures for the overall condition of the fish. The exposure to ALAN lasted 2 weeks (according to Brüning *et al*., 2015), and a large range of nocturnal light intensities were tested because there is little information on the sensitivity of the immune system of fish to ALAN. Intensities of nocturnal illumination ranged from very low typical skyglow intensities of 0.01 lx to 1 lx in one experiment or, in a separate experiment, from 1 lx up to very extreme ALAN intensities of 100 lx that would occur only locally close to a strong streetlight (Hänel *et al*., 2018).

The hypotheses of this study are based on the findings described above in which exposure to continuous illumination or long-day photoperiod led to a modulated immune status and an increase in oxidative stress in different fish species. Moreover, the hypotheses are indirectly based on immuno-enhancing and antioxidative effects of melatonin, which is reduced under ALAN, as it has previously been shown in *P. fluviatilis* with a similar set-up as in the present study (Brüning *et al*., 2015; Kupprat *et al*., 2020). The authors expected a reduction in respiratory burst activity of head kidney leucocytes and increased lysozyme activity as well as an increased lipid peroxidation and an increase in SOD and CAT activities in the liver as indirect measures for an increase in oxidative stress.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The care and use of experimental animals complied with German animal welfare laws, guidelines and policies as approved by the Berlin State Office of Health and Social Affairs (LAGeSo reference number G0055/16).

2.2 | Experimental fish

The authors studied *P. fluviatilis* (pubertal and young adults) from Lake Müggelsee (Berlin, Germany). All experimental fish were kept in 600 l indoor tanks at 16°C for at least 2 weeks before the animals were transferred to the experimental set-up. During this time, fish experienced natural photoperiod (sunlight through windows) with natural dark nights and were fed twice a day with the food source of the respective experiment (see below). According to the "new world atlas of artificial night sky brightness," the surface of Lake Müggelsee experiences an illumination of *c*. 0.003 lx in moonless clear nights (Falchi *et al*., 2016), which lies in the lower range of suburban skyglow (Hänel *et al*., 2018).

2.3 | Experimental set-up

The experimental set-up has been described in detail by Franke *et al*. (2013). Fish were exposed to ALAN treatments in 80 l aquaria, which were covered with black foil to ensure independence of the light treatments. The lids of all aquaria were equipped with three fluorescent tubes to realize daylight intensities that reached up to 7000 lx at the brightest spot on the water surface and around 2900 lx averaged over 25 equally distributed points on the water surface. An additional fluorescent tube was installed for night-time illumination. Control levels were below the detection limit of the luxmeter (ILT1700, Peabody, MA, USA) used in this study, *i.e*. <0.00167 lx, and are referred to as "0 lx" in the following. Photoperiod was controlled by an automatic time switch system (Hager, Blieskastel, Germany). The spectral composition of the light source (Biolux fluorescent tubes, Osram, Germany) has been reported by Franke *et al*. (2013) and can be used to convert lx values into other illumination units (*e.g*. 1 lx ≈ 3.7 mW m^{-2}). It covers a large part of the spectral sensitivity of *P. fluviatilis* although their spectral sensitivity is slightly more red-shifted (Cameron, 1982). Light intensity was adjusted by partial cover of the light source or using neutral density filter foil (Lee Colour Filter 299 1.2 N.D.). Because these methods do not change the spectral composition of the light source, lux can be used as the unit of illuminance for comparison across different light intensities.

2.3.1 | Exposure to high intensities of ALAN ("high ALAN experiment")

Parasite-free *P. fluviatilis* were raised from fertilized egg ribbons collected from Lake Müggelsee (Berlin, Germany) in March 2015 as described by Vivas Muñoz *et al*. (2019). The experimental set-up consisted of 12 identical 80 l aquaria with a tap water flow-through of 10 l h^{-1} and a water temperature of *c*. 16°C. Six fish were placed into each aquarium and allowed to acclimate for 2 weeks without illumination during night (0 lx) followed by 2 weeks of experimental conditions with the respective nocturnal light intensity or controls without illumination according to Brüning *et al*. (2015). Average mass of the fish was 69.0 ± 18.4 g with an average standard length of 15.3 ± 1.3 cm (mean ± S.D., *N* = 120). Fish were fed with commercially available food (Aller Silver 3 mm, Emsland-Aller Aqua, Golßen, Germany) twice a day at a rate of 0.5% of their body mass. Feeding stopped 24 h before sampling. Full daylight was realized from 09:00 to 15:00 hours with a simulated dawn or dusk period over 3 h each starting at 06:00 or 15:00 hours, respectively. Nocturnal illumination with 1 lx, 10 lx or 100 lx on the water surface was from 18:00 till 06:00 hours. The experiment was run twice in December 2016 and January 2017, with each treatment in duplicate during the first run and in triplicate during the second run (*i.e. N* = 5 for each treatment).

2.3.2 | Exposure to low intensities of ALAN ("low ALAN experiment")

P. fluviatilis from Lake Müggelsee were caught between July and September 2017 and fed twice a day with frozen blood worms. Thirty fish with a mass of 16.8 ± 4.1 g and standard length of 10.6 ± 0.9 cm (mean ± S.D., *N* = 720) were transferred to each 80 l aquarium. During 2 weeks of acclimation, fish were fed twice a day and the water flow-through was adjusted to 10 l h^{-1}. The temperature during acclimation and experimental exposure to ALAN was kept around 16°C. Photoperiod was adjusted to October conditions with full daylight from 09:30 to 18:30 hours with a 3 h dawn or dusk period starting at 06:30 and 18:30 hours, respectively. After acclimation, the nocturnal illumination from 21:30 to 6:30 hours was switched on with 0.01 lx, 0.1 lx or 1 lx average intensity on the water surface for 2 weeks according to Brüning *et al*. (2015). Controls were not illuminated during night (0 lx). During experimental illumination, the water flow-through was reduced to 4 l h^{-1} to allow water-based melatonin measurements, which are described by Kupprat *et al*. (2020). To maintain good water quality, animals were not fed during the 2 weeks of exposure. The same experiment was performed twice – in October and November 2017 – with each treatment in triplicates for both runs (*i.e*., *N* = 6 for each treatment).

2.4 | Sampling

Fish were randomly sampled on two consecutive mornings between 09:00 and 12:00 hours in the high ALAN experiment and in two consecutive nights between 22:00 and 04:00 hours in the low ALAN experiment. In the high ALAN experiment, all parameters were measured from all fish. In the low ALAN experiment, body mass and length were measured of all fish, but blood was only taken from the first 15 fish of each aquarium, and the first 10 fish were killed for sampling of livers as well as spleens. Males (m) and females (f) were distinguished by a visual inspection of the gonads. In premature fish (nd) gonads could not be differentiated. Sex ratios were 34%:37%:29% (f:m:nd) in the high ALAN experiment and 5%:57%:38% (f:m:nd) in the low ALAN experiment. In the low ALAN

experiment, sex could be determined only for fish that were killed, and thus this information is not available for some blood samples (na).

Blood (500–1000 μl) was taken from the caudal vein with heparinized syringes and transferred to a tube containing *c*. 1–2 mg Na_2EDTA (EDTA). Blood and EDTA were mixed by shaking and centrifuged at 7500 × *g* for 5 min at 4°C. The plasma was transferred to a new tube and immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Fish were stunned by a blow to the head and killed by cutting the neck. Wet body mass was measured to the nearest 0.1 g and standard length with an accuracy of 1 mm. Fish were then cut open dorsally, and the liver and spleen were excised and weighed to an accuracy of 1 mg. The liver was immediately frozen in liquid nitrogen and stored at −80°C until further analysis. In the low ALAN experiment, 68% of the dissected fish had plerocercoids of *Triaenophorus nodulosus* (Pallas 1781) in the liver, which were excised before freezing. Only non-infected livers were used for calculations of the hepato-somatic index.

Finally, the head kidneys were excised and squeezed through a 70 μm cell sieve (EASYstrainer™, Greiner Bio One International, Kremsmünster, Austria) by adding ice-cold washing medium (RPMI 1640 medium with phenol red with 25 mM HEPES, 100 U ml^{-1} penicillin–streptomycin, 2.05 mM L-glutamine, Biowest, Nuaillé, France and 20 IU ml^{-1} heparin) and stored on ice until the end of sampling.

If not indicated otherwise, chemicals were obtained from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

2.5 | Respiratory burst activity

The respiratory burst activity assay was based on the method originally described by Secombes (1990) and performed following the protocol by Liu *et al*. (2017) with some modifications. Cell cultures were only prepared in the high ALAN experiment. After sampling, cells were centrifuged at 500 × *g* for 15 min at 4°C, and the volume of the suspension was adjusted to 5 ml. Density gradient centrifugation was used to enrich macrophages and to separate them from erythrocytes: The cell suspension was layered on a discontinuous gradient consisting of 51% and 34% Percoll (GE Healthcare, Chicago, IL, USA) and centrifuged at 500 × *g* for 40 min at 4°C. Cells collected from the 34–51% interphase were washed once in washing medium and once with cell culture medium (RPMI 1640 with 25 mM HEPES, 100 U ml^{-1} penicillin–streptomycin, 2.05 mM L-glutamine, Biowest) by centrifugation at 500 × *g* for 15 min at 4°C and re-suspended in cell culture medium. The cell concentration was adjusted to 10^7 cells ml^{-1} and 100 μl of this suspension was added to eight wells of a cell culture-treated 96-well plate (Nunclon® Surface, Thermo Scientific, Waltham, MA, USA). Cells were allowed to adhere for 1 h at room temperature. Non-adherent cells were then removed by carefully rinsing with 150 μl cell culture medium. Subsequently, cells were incubated for 1 h at 25°C in culture medium containing 1 mg ml^{-1} nitroblue tetrazolium chloride, and half of the cells were stimulated with 1 μg ml^{-1} phorbol 12-myristate 13-acetate (PMA). Cells *without stimulation* served as control. Finally, cell layers were washed again with 150 μl of cell culture medium and fixed in 100 μl methanol, and then washed twice with 100 μl of 70% methanol and air-dried. The intracellularly produced formazan was dissolved with 100 μl of 2 M KOH and 100 μl of DMSO and mixed thoroughly. Absorption was measured at 620 nm.

2.6 | Lysozyme activity

Lysozyme activity was measured according to Ellis (1990) adapted to a 96-well plate and optimized for plasma of *P. fluviatilis*. Plasma samples were diluted 1:2 with 0.025 M potassium sodium phosphate buffer pH 6.2, and 25 μl of diluted sample was added to 175 μl *Micrococcus lysodeikticus* suspension (0.2 mg lyophilized bacteria in 1 ml of the same buffer). After the sample was shaken for 5 min at 21°C on an orbital shaker at 300 rpm, the optical density was measured at 530 nm every minute over 15 min at 25°C. A linear regression was calculated for each sample for the time interval between 7 and 12 min, at which the decline was linear in all samples. According to Ellis (1990), a decline of 0.001 min^{-1} was defined as one unit of lysozyme activity.

2.7 | Liver extracts

As an indirect measure for oxidative stress, two antioxidative enzymes and one indicator for lipid peroxidation were measured from liver tissue. In the high ALAN experiments all liver samples were analysed. For the low ALAN experiment six liver samples were randomly chosen from the 10 fish sampled from each aquarium. Livers were homogenized manually with a glass homogenizer by adding sodium phosphate buffer pH 7.0 with 0.5 M EDTA at 1 ml per 0.1 g liver mass. A 300 μl aliquot of this homogenate was taken for analysis of TBARS and stored at −80°C until assayed. Another millilitre of the homogenate was centrifuged at 10,000 × *g*, and the supernatant was aliquoted for analyses of protein, SOD and CAT and stored at −80°C until assayed.

2.7.1 | Thiobarbituric acid reactive substances

Lipid peroxidation was measured with the TBARS assay based on the procedure described by Uchiyama and Mihara (1978) with a standard curve of tetraethoxypropane ranging from 1 to 50 nmol ml^{-1}. To lyse fatty acids from the liver homogenate, 250 μl of samples and standards were mixed with 250 μl of 7% SDS solution and incubated for 5 min at room temperature. After putting samples on ice and adding 500 μl of 12.5% TCA in 0.8 M HCl, 500 μl of 1% thiobarbituric acid (TBA) was added and the mixture was heated to 95°C for 45 min. Under these conditions, TBA and malondialdehyde from samples or standards reacted to produce a pink-coloured dye, which was extracted with 1500 μl of

1-butanol by vortexing for 1 min followed by centrifugation at 4500 × *g* for 10 min at 4°C. The absorbance of the supernatants was measured in triplicates at 535 nm.

2.7.2 | Superoxide dismutase

The activity of SOD was measured with a commercially available kit (Item 706002, Cayman Chemicals, Ann Arbor, MI, USA). Liver extracts were diluted 1:200 to 1:400 with "assay sample buffer" from the kit and measured according to the manufacturer's protocol. Before measuring all samples, the authors measured three samples at dilutions of 1:100, 1:200 and 1:400, and the calculated, original concentrations did not differ more than 20% from each other. Activities were quantified by a standard curve of bovine SOD covering the range from 0.005 to 0.05 U ml^{-1}.

2.7.3 | Catalase

The activity of CAT was measured according to the protocol of Aebi (1984) adapted to a 96-well format. The ratio of the reaction media was adjusted for this study's samples. Liver extracts were diluted 1:50 with 50 mM sodium phosphate buffer pH 7.0. First, 180 µl of buffer was added to a 96-well plate (UV-star, Greiner Bio One International, Kremsmünster, Austria), and 15 µl of 150 mM H_2O_2 solution (prepared in the same buffer) was added. Then, 5 µl of diluted sample was added to the mixture, the plate was shaken for 5 s at 500 rpm at 25°C and then the absorption was measured at 240 nm every 30 s for 10 min at 25°C. The linear regression of decreasing absorption had R^2 > 0.8 in all analysed samples. CAT activity is expressed as units (U), which equals 1 µmol of H_2O_2 consumed per minute with an extinction coefficient for H_2O_2 of 43.6 M^{-1} cm^{-1} and a path length of 0.5 cm.

2.7.4 | Liver protein content

To normalize SOD and CAT activities, the total protein concentration in the liver extracts was measured with the Biuret reaction using a commercially available kit (RotiQuant® Universal, Roth, Karlsruhe, Germany). Quantification was made by a standard curve of bovine serum albumin.

2.8 | Body indices: condition factor, hepato-somatic index and spleno-somatic index

To assess rough measures for the overall health of the fish, three body indices were calculated. The condition factor was calculated by $K = 100\ M_W\ L_S^{-3}$, where M_W (g) is the wet mass and L_S (cm) is the standard length. The hepato-somatic index was calculated by $I_H = 100\ M_l\ M_W^{-1}$, where M_L (g) is the liver mass and the spleno-somatic index was calculated likewise by $I_S = 100\ M_S\ M_W^{-1}$, where M_S (g) is the spleen mass.

2.9 | Statistical analysis

A linear mixed modelling (LMM) approach was chosen to account for the data structure with treatment and sex as fixed factors and individuals nested within aquaria nested within runs as random factors (R Core Team, 2020; Zuur *et al*., 2009). For the respiratory burst activity, stimulation was added as a fixed factor, and interaction with treatment was tested. Statistical significance was assumed with $P < 0.05$. In case of significant treatment or sex effects, *post hoc* tests using Bonferroni's correction compared every treatment to every other treatment within one experiment. Treatment, sex and random effects as well as marginal and conditional R^2 values for each LMM are specified in Table 1. Full model specifications for each parameter and each experiment as well as full results of the *post hoc* tests are given in Supporting Information. In addition, effects of liver parasitization were tested by comparing infected to non-infected individuals by Mann–Whitney *U*-tests. R packages used for statistical analysis and data visualization are listed in Supporting Information.

2.10 | Note on the differences between the two experiments

Because of the differences in size and life history of the fish as well as differences in feeding regimes and different sampling times among the two experiments, the authors only compare the measured parameters within one experiment and not across the low and high ALAN experiments. Originally, the authors aimed to do the high ALAN experiment with a wild population from Lake Müggelsee as it was later realized in the low ALAN experiment and has been realized in an earlier study (Brüning *et al*., 2015). Nonetheless, in fall 2016, the authors were unable to catch sufficient wild *P. fluviatilis* for the experiment and therefore decided to work with lab-raised fish, which were conditioned to dry feed. Contrarily, the wild *P. fluviatilis* in the low ALAN experiment could not be conditioned to dry feed and were thus fed with frozen blood worms. As mentioned earlier in Section 2.3.2, fish in the low ALAN experiment were not fed during the experimental time because of low water flow-through to measure melatonin from the aquarium water (Kupprat *et al*., 2020). This was not necessary in the high ALAN experiment because changes in nocturnal melatonin were known from a previous study (Brüning *et al*., 2015). In the high ALAN experiment, sampling occurred in the morning after fish were exposed to ALAN for the whole night. When the authors measured only few differences between the treatments in the high ALAN experiment, they supposed that effects at even lower intensities at the same time of day would be unlikely, and therefore, they took the samples throughout the night in the low ALAN experiment.

TABLE 1 Effects of artificial light at night (ALAN) and sex as well as random effects and the marginal and conditional R^2 values for the linear mixed models of different immune parameters, proxies of oxidative stress and body indices in two different experiments exposing *Perca fluviatilis* to different nocturnal light intensities for 2 weeks

	ALAN effect		Sex effect		Random effects		Goodness of fit	
High ALAN experiment	**LLR[a]**	***P*-value**	**LLR**	***P*-value**	**LLR**	***P*-value**	$R^2_{marginal}$	$R^2_{conditional}$
Respiratory burst activity	2.05	0.56	5.96	0.051	400.03	<0.0001	0.9955	0.9988
Lysozyme activity	3.07	0.38	6.15	0.046[b]	621.02	<0.0001	0.0684	0.9511
Thiobarbituric acid reactive substances	7.61	0.055	59.77	<0.0001	1237.5	<0.0001	0.4172	0.9990
Liver protein	1.61	0.66	14.22	0.0008	217.71	<0.0001	0.1154	0.9355
Superoxide dismutase activity	1.61	0.63	5.27	0.07	138.38	<0.0001	0.0584	0.9798
Catalase activity	1.73	0.63	15.06	0.0005	260.88	<0.0001	0.1071	0.9308
Condition factor	3.64	0.30	6.01	0.0495[b]	0.05	0.97	0.0929	0.1142
Spleno-somatic index	3.21	0.36	11.39	0.003	1.62	0.44	0.0409	0.0751
Hepato-somatic index	8.93	0.03	20.49	<0.0001	9.61	0.008	0.1943	0.3762
Low ALAN experiment	**LLR**	***P*-value**	**LLR**	***P*-value**	**LLR**	***P*-value**	$R^2_{marginal}$	$R^2_{conditional}$
Lysozyme activity	1.66	0.64	6.44	0.09	275.87	<0.0001	0.0308	0.9290
Thiobarbituric acid reactive substances	0.38	0.94	29.42	<0.0001	1003.6	<0.0001	0.1710	0.9914
Liver protein	1.21	0.75	1.49	0.48	432.32	<0.0001	0.0120	0.9923
Superoxide dismutase activity	1.68	0.64	9.68	0.008	108.01	<0.0001	0.0788	0.9017
Catalase activity	0.55	0.91	0.61	0.73	304.48	<0.0001	0.0067	0.7895
Condition factor	1.40	0.71	45.05	<0.0001	209.44	<0.0001	0.0366	0.4522
Spleno-somatic index	2.36	0.50	19.89	0.0001	6.32	0.04	0.1384	0.2815
Hepato-somatic index	2.25	0.52	25.92	<0.0001	6.1^{-9}	1	0.1422	0.1422

[a]Log-likelihood ratio.
[b]Significant effect in model selection, but no significant differences in *post hoc* testing.

3 | RESULTS

3.1 | Respiratory burst activity

The respiratory burst was strongly stimulated by PMA in all cultures of head kidney leucocytes in the high ALAN experiment (LMM: fixed factor stimulation: $P < 0.0001$; Figure 1). Random effects explained a significant proportion of the variance that was not explained by the fixed effect ($P < 0.0001$, Table 1), mainly because of individual variation (high ALAN: $\sigma^2_{ind} > \sigma^2_{aqu} > \sigma^2_{run}$). There were no significant differences between sexes in respiratory burst activity.

3.2 | Lysozyme activity

Antibacterial activity of lysozyme in the blood plasma was measured as a humoral factor of the innate immune system and did not differ across treatments in both experiments (Figure 2; Table 1). There were no significant random effects in either experiment as well as no significant differences between sexes in lysozyme activity ($P > 0.05$, Table 1). Although in the model selection a sex effect for the high ALAN experiment was suggested ($P = 0.046$, Table 1), *post hoc* testing did not reveal significant differences ($P > 0.05$). Parasitization in the liver did not affect lysozyme activity in the low ALAN experiment (Mann–Whitney *U*-test, $P = 0.51$, Supporting Information).

3.3 | Oxidative stress in the liver: TBARS, SOD, CAT and liver protein

The indirect measures for oxidative stress, measured by the TBARS assay from liver homogenate (Figure 3) and by SOD and CAT activities in liver extracts (Figure 4), did not differ significantly across treatments of different nocturnal illumination in both experiments ($P > 0.05$, Table 1). The protein content in the liver was used to standardize enzyme activities and did not differ significantly across treatments in both experiments, either ($P > 0.05$, Figure 4; Table 1). The graphs for the liver protein are attached in Supporting Information. All LMMs for the liver parameters revealed significant random effects with highest variance of individuals, which explained the biggest portion of the data variance ($\sigma^2_{ind} > \sigma^2_{run}$ or σ^2_{aqu}).

All liver parameters had sex effects in at least one experiment (Table 1). Males had higher TBARS levels than females (both experiments: $P < 0.0001$) and premature (nd) fish (high ALAN exp.: $P < 0.0001$, low ALAN exp.: $P = 0.003$). In addition, in the low ALAN experiment premature fish had higher levels of TBARS in the liver than females ($P = 0.005$). The protein content in the liver was higher in premature fish than in males or females in the high ALAN experiment ($P < 0.0001$), and in the low ALAN experiment there were no significant sex effects ($P = 0.48$, Table 1). The activity of SOD showed no sex effects in the high ALAN experiment ($P = 0.07$), but in the low ALAN experiment the premature fish had higher activities than males

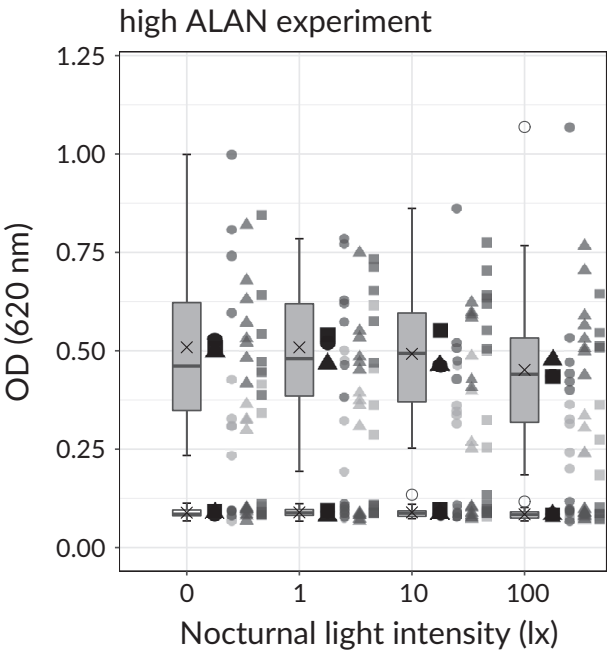

FIGURE 1 Respiratory burst activity in head kidney leucocytes of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks. Boxplots display non-stimulated cells (white boxplots) and cells stimulated with phorbol 12-myristate 13-acetate (PMA; grey boxplots) for each treatment (high ALAN experiment: $N = 30$). Optical density (OD) was determined at 620 nm. Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers >1.5 × IQR are indicated by circles and Xs inside the boxes indicate the mean. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)]. Small points represent individual cell cultures, whereas different shades of grey indicate different runs. There were no significant differences across treatments [linear mixed model, ALAN effect: log-likelihood ratio (LLR) = 2.05, $P = 0.56$] (□) no stimulation (■) PMA stimulation (●) December (●) January (●) f (▲) m (■) nd

or females (nd *vs*. m: $P = 0.03$, nd *vs*. f: $P = 0.047$). The activity of CAT was higher in males of the high ALAN experiment than in females or premature fish (m *vs*. f: $P = 0.0005$, m *vs*. nd: $P = 0.047$), but had no sex effect in the low ALAN experiment ($P = 0.74$, Table 1). Parasitization in the liver did not affect any of the hepatic parameters in the low ALAN experiment (Mann–Whitney *U*-test, $P > 0.05$, Supporting Information).

3.4 | Body indices

There were no significant effects across the different nocturnal illuminations both in *K* and I_S of either experiment or in the I_H of the low ALAN experiment ($P > 0.05$, Table 1, Figure 5). The graphs for *K* are attached in Supporting Information. At 100 lx, I_H was significantly lowered by 15% compared to 0 lx in the high ALAN experiment (LMM, treatment as fixed effect: $P = 0.0303$, Tukey's *post hoc*: $P = 0.046$).

The random effects were significant for I_H in the high ALAN experiment in which run had a higher variance than aquarium ($\sigma^2_{run} > \sigma^2_{aqu}$) and for *K* and I_S of the low ALAN experiment in which run or aquarium had a higher variance, respectively (*K*: $\sigma^2_{run} > \sigma^2_{aqu}$; I_S: $\sigma^2_{aqu} > \sigma^2_{run}$).

Sex had a significant effect on all body indices in both experiments ($P < 0.05$, Table 1). Nonetheless, *post hoc* tests revealed no significant differences between sexes in *K* of the high ALAN experiment. In the low ALAN experiment, premature fish had a lower *K* than males or females (nd *vs*. f: $P = 0.0156$; nd *vs*. m: $P < 0.0001$). I_S was higher in premature fish than in males in both experiments (high ALAN exp.: $P = 0.0088$; low ALAN exp.: $P < 0.0001$). I_H was higher in females than in premature or male fish in both experiments (f *vs*. m: $P < 0.0001$; f *vs*. nd (high ALAN exp.): $P < 0.0117$; f *vs*. nd (low ALAN exp.): $P < 0.0001$). The I_S was significantly lower (*c*. 6%) in fish infected with liver parasites compared to fish without infected livers (Mann–Whitney *U*-test, $P = 0.01$, Supporting Information).

4 | DISCUSSION

This study aimed to assess the effects of ALAN at illuminances between 0.01 lx and 100 lx on the innate immune system, indicators for oxidative stress and body condition of *P. fluviatilis* after 2 weeks. In contrast to most of the hypotheses of this study, the measured parameters remained largely unchanged at all tested levels of ALAN. Nonetheless, there was a significant decrease in the hepato-somatic index (I_H) at the highest ALAN level of 100 lx compared to 0 lx.

4.1 | Respiratory burst activity

The respiratory burst activity – a parameter for innate cellular immunity – showed no significant changes in response to 1 lx, 10 lx or 100 lx of nocturnal illumination. This lack of effects of high ALAN intensities on respiratory burst activity contrasts results from studies with continuous illumination or long-day photoperiod in *O. mykiss* or *C. carpio* (Burgos *et al*., 2004; Kepka *et al*., 2015). The natural variation in respiratory burst activity among individuals might have been too broad to detect effects of ALAN in *in vivo* experiments after only 2 weeks. Significant changes in respiratory burst activity in *in vivo* experiments were measured in experiments, which lasted 4–8 weeks and had harmful or stimulating additives in the fish food as a treatment (*e.g*., Adel *et al*., 2016; Pietsch *et al*., 2014), which might modulate the immune system more effectively than ALAN. Furthermore, ALAN or indirectly the reduced melatonin might come into effect only in combination with other factors. Accordingly, in leucocytes of *C. carpio* melatonin treatment only changed respiratory burst activity *in vivo* in a zymosan-induced peritonitis but did not show enhancing effects at *in vitro* treatments with different melatonin concentrations (Kepka *et al*., 2015).

4.2 | Lysozyme activity in blood plasma

Opposing the hypothesis of this study, lysozyme activity was not elevated by any ALAN treatment in the experiments of this study. This

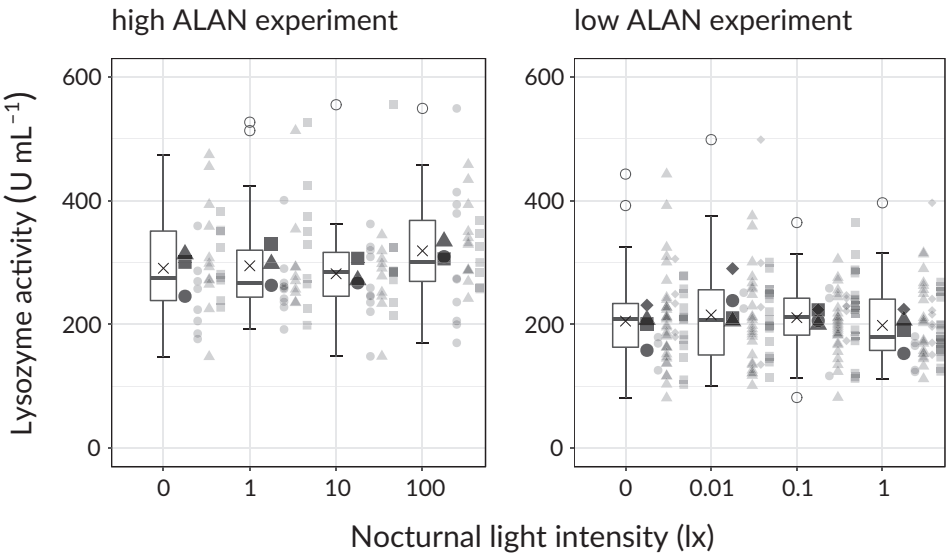

FIGURE 2 Lysozyme activity in the blood plasma of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. Activity is expressed as units (U) per millilitre. Boxplots display data for each treatment and Xs inside the boxes indicate the mean (high ALAN experiment: $N = 29$ for 0 lx and 100 lx, $N = 30$ for 1 lx and 10 lx; low ALAN experiment: $N = 57$ for 0 lx, $N = 49$ for 0.01 lx, $N = 59$ for 0.1 lx, $N = 54$ for 1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers >1.5 × IQR are indicated by circles. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish), na – not available (sex not determined)], whereas small points represent individuals. There were no significant differences across treatments in either experiment [linear mixed models, ALAN effect in high ALAN experiment: log-likelihood ratio (LLR) = 3.07, $P = 0.38$; ALAN effect in low ALAN experiment: LLR = 1.66, $P = 0.64$] (●) f (▲) m (■) nd (◆) na

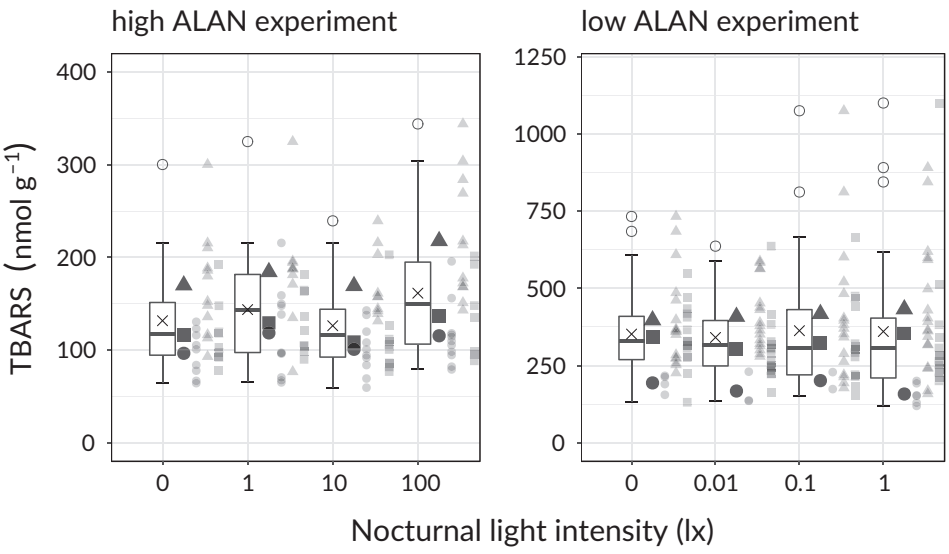

FIGURE 3 Thiobarbituric acid reactive substances (TBARS) of liver homogenate of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. The measurements are expressed as nanomoles malondialdehyde equivalent per gram liver mass. Boxplots display data for each treatment and Xs inside the boxes indicate the mean (high ALAN experiment: $N = 30$; low ALAN experiment: $N = 36$ for 0 lx and 0.01 lx, $N = 34$ for 0.1 lx, $N = 37$ for 1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers >1.5 × IQR are indicated by circles. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)], whereas small points represent individuals. There were no significant differences across treatments in either experiment [linear mixed models, ALAN effect in high ALAN experiment: log-likelihood ratio (LLR) = 7.61, $P = 0.055$; ALAN effect in low ALAN experiment: LLR = 0.38, $P = 0.94$] (●) f (▲) m (■) nd

contradicts previous findings in *O. mykiss* at continuous illumination or long-day photoperiod with elevated lysozyme content after 1 or 4 weeks, respectively (Burgos *et al*., 2004). It is possible that typical ALAN levels have no effect on lysozyme activity of *P. fluviatilis* or an exposure to ALAN may take longer than 2 weeks for lysozyme activity to respond. For example, it was only recently shown that the overall

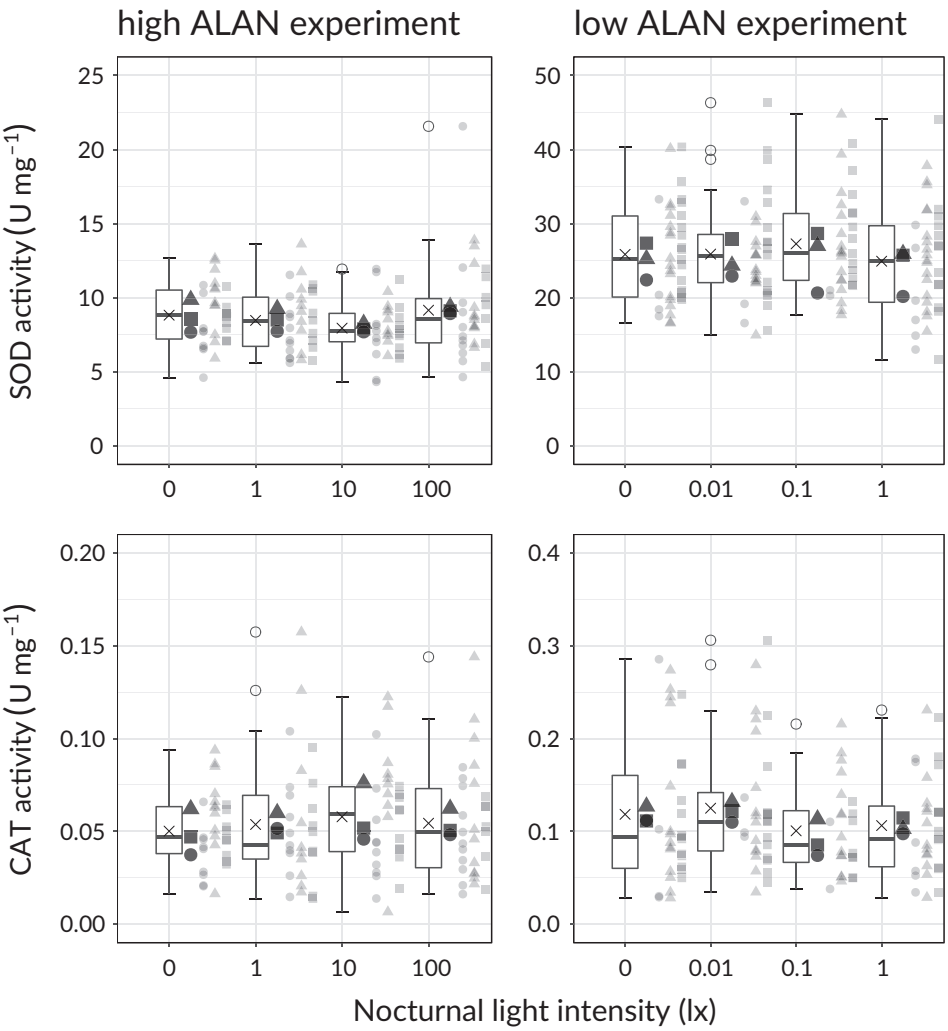

FIGURE 4 Activities of the antioxidative enzymes superoxide dismutase (SOD) and catalase (CAT) measured from liver extracts of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. Specific activities of both enzymes are expressed as units (U) per milligram total liver protein. Boxplots display data for each treatment and Xs inside the boxes indicate the mean (high ALAN experiment – SOD: $N = 30$, – CAT: $N = 30$ for 0 lx, 10 lx and 100 lx, $N = 29$ for 1 lx; low ALAN experiment – SOD: $N = 37$ for 0 lx and 1 lx, $N = 35$ for 0.01 lx, $N = 33$ for 0.1 lx, – CAT: $N = 31$ for 0 lx, 0.01 lx and 1 lx, $N = 24$ for 0.1 lx). Boxplots are limited by the 25% and 75% quartile, with a horizontal line as the median and whiskers depicting the 1.5 × interquartile ranges (IQR); outliers >1.5 × IQR are indicated by circles. Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)], whereas small points represent individuals. There were no significant differences across treatments in either experiment [linear mixed models, ALAN effects in high ALAN experiment: SOD – log-likelihood ratio (LLR) = 1.61, $P = 0.63$, CAT – LLR = 1.73, $P = 0.63$; ALAN effect in low ALAN experiment: SOD – LLR = 1.68, $P = 0.64$, CAT – LLR = 0.55, $P = 0.91$] (●) f (▲) m (■) nd

bacterial-killing activity in blood plasma of king quail *Excalfactoria chinensis* (L. 1766) was increased only after 4 or 6 weeks exposure to weak ALAN (0.3 lx) in developing females and males (Saini *et al*., 2019).

4.3 | Oxidative stress in the liver

The TBARS and the activities of SOD and CAT in liver extracts did not significantly change after 2-week exposures to ALAN, suggesting that there were no substantial changes in oxidative stress in this tissue. Similar to the results of this study, the oxidative status in wild songbirds was not affected by ALAN at an illuminance level of 3 lx, although body mass changed after 2 days of exposure (Raap *et al*., 2016). Yet, some antioxidant capacity was likely missing in the experimental fish of this study, as it was shown in earlier publications that melatonin concentrations decreased in *P. fluviatilis* under ALAN in a dose–response manner (Brüning *et al*., 2015; Kupprat *et al*., 2020). Probably, other antioxidative mechanisms compensated for the lacking melatonin without a measurable increase in activity of SOD and CAT. For further testing of the hypothesis that a lack of melatonin can lead to an increase in oxidative stress, the combination of ALAN with another stressor, *e.g*., increased temperature, might be a promising approach to evaluate how the antioxidative system of fish

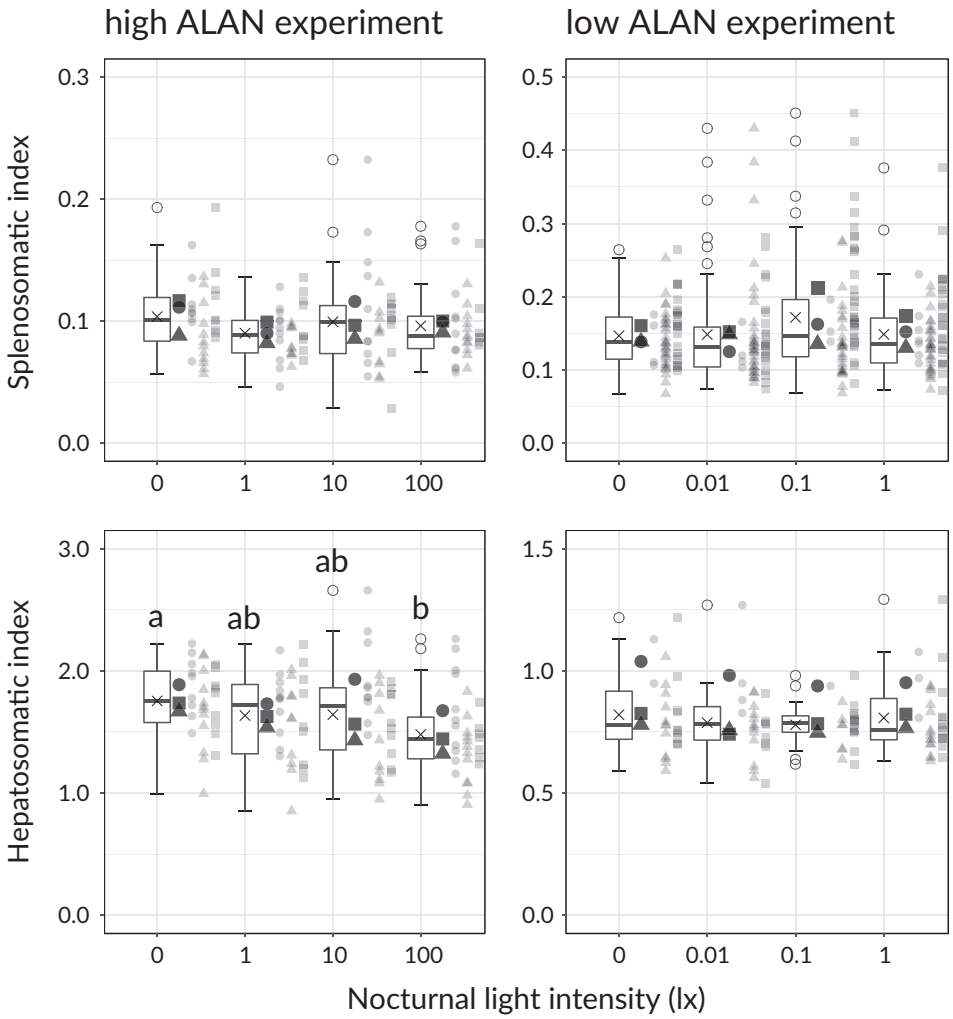

FIGURE 5 Spleno-somatic index (I_S) and hepato-somatic index (I_H) of *Perca fluviatilis* exposed to different intensities of artificial light at night (ALAN) for 2 weeks in two different experiments. Boxplots display data for each treatment, and Xs inside the boxes indicate the mean (high ALAN experiment: $N = 30$; low ALAN experiment – I_S: $N = 63$ for 0 lx, $N = 64$ for 0.01 lx, $N = 65$ for 0.1 lx and 1 lx, – I_H: $N = 24$ for 0 lx, $N = 25$ for 0.01 lx, $N = 22$ for 0.1 lx, $N = 31$ for 1 lx). Different shapes of big points indicate the mean of each sex [m – males, f – females, nd – not differentiated (premature fish)], whereas small points represent individuals. Letters indicate a significant decrease at 100 lx as compared to 0 lx (Tukey's *post hoc*, $P = 0.0457$) for the I_H in the high ALAN experiment. There were no significant differences across treatments in the I_S in either experiment [linear mixed models, ALAN effect in high ALAN experiment: I_S – log-likelihood ratio (LLR) = 3.21, $P = 0.36$, I_H – LLR = 8.93, $P = 0.03$; ALAN effect in low ALAN experiment: I_S – LLR = 2.36, $P = 0.50$, I_H – LLR = 2.25, $P = 0.52$] (●) f (▲) m (■) nd

responds to ALAN under realistic oxidative stress. Furthermore, *in vitro* experiments exposing isolated cells or tissues to oxidative stress with simultaneous melatonin treatment might also help understanding the mechanisms of melatonin and its contribution to the antioxidative capacity in fish tissues. A similar approach was used by Sreejith *et al.* (2007) in climbing perch *Anabas testudineus* (Bloch 1792) in which oxidative stress was induced by 6-propylthiouracil (PTU) treatment or continuous illumination. In cultivated liver tissue of *A. testudineus*, melatonin reduced the lipid peroxidation caused by continuous illumination and reduced the increased activities of, *e.g.*, SOD and CAT in combination with 6-PTU treatment even below control levels, whereas 6-PTU alone increased activities of SOD and CAT. The results therefore suggest a strong contribution of melatonin to the antioxidative capacity in fish.

4.4 | Body indices: I_S and I_H

The I_S as an indicator for haematopoietic and immunological activity did not change in the experiments of this study. In contrast, as in small rodents short-day photoperiods increased splenic mass (*e.g.* Vaughan *et al.*, 1987), decreased splenic mass might be expected at long-day photoperiods or continuous light. The results of this study, however, showed that the splenic mass of *P. fluviatilis* is not affected by ALAN between 0.01 and 100 lx within 2 weeks. Irrespective of the ALAN treatment, infections with the liver parasite *T. nodulosus* slightly lowered relative splenic mass as compared to non-infected *P. fluviatilis*. *T. nodulosus* is a common parasite of the Northern pike *Esox lucius* L. 1758 and frequently infects *P. fluviatilis* as an intermediate host in which most worms encapsulate in the liver. Although most other parameters reported here were not affected by the liver parasite, the lowered I_S may indicate a weak suppression of haematopoietic and immune function of the spleen.

The significant decrease in I_H at 100 lx can be interpreted as an indicator of decreased energy storage, such as glycogen reserves (Chellappa *et al.*, 1995). Interestingly, there seems to be an inverse trend of I_H and TBARS at 100 lx with decreased I_H and increased TBARS, despite a *P*-value closely above the threshold of statistical significance for the latter one (Table 1). A confirmation of this inverse relationship at high intensities of ALAN is necessary before further conclusions about the effects of ALAN on the liver metabolism of fish can be drawn. In contrast, other studies rather indicate a positive relationship between TBARS and I_H in fish (*e.g.*, Chien & Hwang, 2001). Further experiments with a longer exposure time and subsequent

analysis of the caloric composition of protein, lipid and carbohydrate in the liver could reveal further insights into the effects of ALAN on liver metabolism. Such experiments should take sex-dependent differences into account because of differences in I_H and TBARS across sexes in the results of this study.

If the decrease in I_H results from a decrease in energy storage such as glycogen, future studies should also address the processes leading to an increased energy demand. For example, increased locomotor activity and concomitant increased oxygen consumption could lead to a higher energy demand in general or to increased oxidative stress. For example, farmed Atlantic salmon *Salmo salar* L. 1758 showed continuous swimming activity at night under continuous light as compared to reduced activity in dark nights of natural photoperiod (Oppedal *et al*., 2001). It is not possible to estimate these complex processes from the results of this study, but the liver metabolism is probably of interest in future ALAN experiments.

4.5 | General discussion of hypotheses

Most of the initial hypotheses were not verified by the experiments of this study. Therefore, based on the current results of this study, new hypotheses and follow-up experiments are needed to further investigate the effects of ALAN on the immune system and antioxidative responses. In the following, the authors discuss explanations for why the hypotheses were not verified and formulate research questions for future research.

Firstly, the direct conclusion would be that ALAN has no effect on the measured parameters of the immune system and indicators for oxidative stress in *P. fluviatilis*. Negative effects of ALAN could be compensated by means that were not measured in this study. *P. fluviatilis* is an euryoecious species that is known for its high potential for adaptation to a wide range of environmental conditions, which may be one of the reasons of its wide distribution range, and a robust immune system might be a key factor in this ability to adapt to a wide range of environmental conditions. There is hardly any information about adaptation potential of animals to ALAN. Because the origin of the experimental animals is only slightly light polluted (Lake Müggelsee experiences horizontal illuminance levels because of skyglow of probably *c*. 0.005 lx, Jechow *et al*., 2020), the authors do not assume that a lack of effects is because of adaptation of the experimental animals to the low intensities of skyglow. Nonetheless, if the immune system and the oxidative status of *P. fluviatilis* withstand ALAN effects, other fish species might be less flexible in adjusting to ALAN. For example, species-specific effects of continuous illumination were shown in early life stages of four different freshwater species (Brüning *et al*., 2010). ALAN scenarios, however, might not be as comparable with scenarios of continuous illumination or long-day photoperiods as assumed. Even at extremely light polluted locations, days are still orders of magnitudes brighter than nights and therefore physiological rhythms may be maintained under ALAN but not under continuous illumination depending on the intensity of nocturnal illumination. For example, rhythmicity of melatonin secretion in *P. fluviatilis* was only depleted at nocturnal intensities above 10 lx (Brüning *et al*., 2015), whereas lower intensities of ALAN below 10 lx led to strongly reduced levels of nocturnal melatonin but rhythmicity was maintained (Brüning *et al*., 2015; Kupprat *et al*., 2020). In studies with long-day photoperiod, animals still experience dark nights but with a shortened scotophase. Thus, the hypothesized effects based on reduced melatonin may not come into effect because an intact rhythmicity of melatonin is still present despite changes in the amplitude.

Secondly, the duration of exposure may be critical for the consideration of responses to ALAN. To better predict the physiological implications of ALAN, it would be critical to also include long-term processes because ALAN may elicit rather slowly increasing responses such as physiological and behavioural compensatory mechanisms (Gaston *et al*., 2015). Similar to negative implications of lacking vitamin C in humans (*e.g*., Pohanka *et al*., 2012), it is possible that changes because of reduced melatonin will come into effect only after longer exposure times, *i.e*., several months. Nonetheless, short-term effects after several days with compensatory acclimation processes after 2 weeks are also possible, but continuous monitoring of immune, antioxidative and conditional parameters is necessary to estimate the time scales of these processes.

Lastly, another option to further test the hypotheses of this study even in short-term experiments could be a combination of ALAN with another environmental stressor. Possible factors for multi-factorial experimental designs are increased temperature, reduced pH, reduced food availability or food quality, a combination with typical ecotoxicological stressors (*e.g*., exposure to heavy metals) as well as increased predation pressure or outbreaks of diseases, whereas the latter two are experimentally more sophisticated.

4.6 | Sex effects

The LMMs of lysozyme activity and *K* from the high ALAN experiment suggested significant sex effects but did not have significant differences in the *post hoc* testing. For lysozyme activity and *K* in the high ALAN experiment, *P*-values of the sex effects are closely below the threshold for significance, which might explain why no effects in the *post hoc* test could be resolved.

In general, the results of this study show that there are differences among sexes in some physiological parameters of *P. fluviatilis*, particularly in TBARS, in the activities of antioxidative enzymes and in I_S and I_H, but without sex effects on the innate immune parameters. These results suggest that oxidative stress, haematopoietic and immunological activity of the spleen as well as hepatic energy storage are different across sexes. This underlines the importance of considering sex-dependent differences in future research investigating oxidative stress or body indices in fish.

5 | CONCLUSION

In contrast to the initial hypotheses of this study based on previous research on the effects of continuous illumination or long-day

photoperiod, the results of this study do not indicate that ALAN affects the innate immune system or oxidative stress in *P. fluviatilis* after 2 weeks of exposure. The response parameters did not differ from controls with dark nights at various levels of nocturnal illumination between 0.01 lx and 100 lx in two separate experiments. Still, long-term studies or a combination with other factors such as elevated temperature as well as studies on other fish species should be the subject for future research on fish health with respect to light pollution and circadian rhythms. Because the authors found a significant decrease in the hepato-somatic index at 100 lx, the hepatic metabolism might be of interest for such studies. Further, both short-term and long-term studies are required for a better mechanistic understanding of how ALAN-disrupted behavioural and physiological biorhythms might modulate the immune system and oxidative stress of fish. This will lead to a deeper overall understanding of the potential threats of light pollution to fish.

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AUTHOR CONTRIBUTIONS

F.H., F.K. and W.K. conceptualized the experiments. F.K., K.K. and T.P. planned and optimized the lab analyses. F.K. and T.P. ran the experiments, samplings and laboratory analyses. F.K. did the statistical analysis of the data and wrote the initial draft of the manuscript. All authors contributed to reviewing and editing the manuscript.

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